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13. ABSTRACT (Maximum 200 words)

Work over the trimester focused on developing LBP peptides with higher binding affinities, defining the best BPI binding protein (BPI) peptide sequence, generating peptide-IgG conjugates with this sequence, developing an assay for opsinophagocytosis, and starting work on the evaluation of the pharmacokinetics of the conjugates. We synthesized overlapping peptides of the published sequence of BPI, and identified $BPI_{85\text{-}99}$ as the optimal binding sequence. As in the LBP peptide studies reported earlier, placement of a terminal cysteine on the peptide increased activity. Conjugates of $BPI_{85\text{-}99}$-IgG were generated using SMPT as a linker, and $BPI_{85\text{-}99C}$-IgG was found to bind LPS slightly better than $BPI_{C85\text{-}99}$-IgG. A opsinophagocytosis assay was developed. Clearance studies were started; in preliminary studies a peptide-IgG conjugate based on a CAP18 peptide with high LPS binding activity was studied. These experiments indicated that the T1/2 of this conjugate was greater than 24 hours. Further studies using these assay systems are planned when larger amounts of the LBP/BPI conjugates are available.

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I. Work Summary

We have continued along our initial project plan. This consists of defining the sequences of LPS binding protein (LBP) and bactericidal/permeability increasing protein (BPI) that bind LPS with the highest affinity, synthesizing peptides that consist of these sequences, characterizing the biological properties of these peptides, coupling them to IgG to create hybrid peptide-IgG conjugates, and evaluating the conjugates for protective activity.

Work on specific aim #1

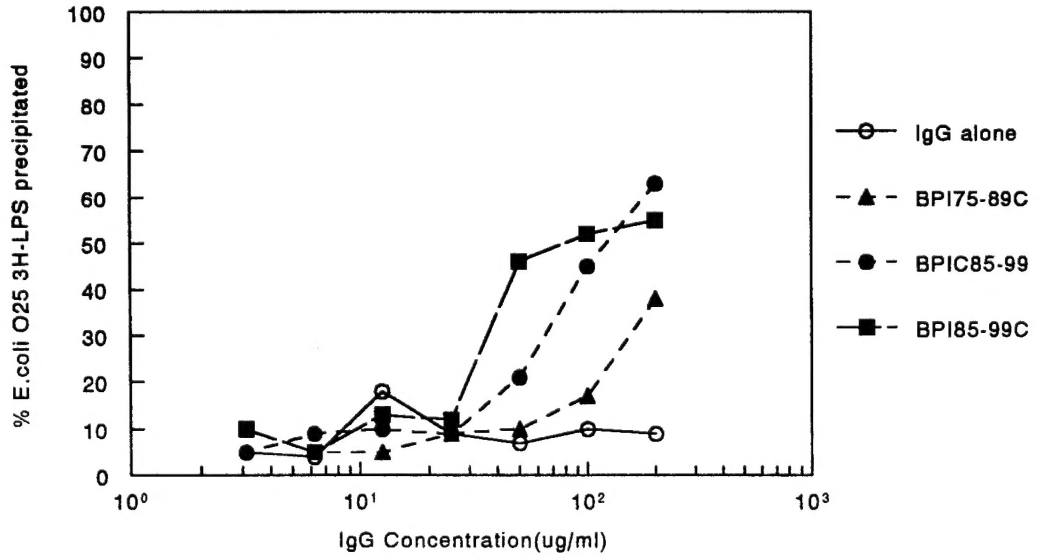
As of the early summer of this year, we had identified the optimal sequence of LBP (LBP_{86-106}). We had radiolabeled this sequence with ^{14}C, created LBP_{86-106}-IgG with 3.9 and 9.5 peptides/IgG and studied the LPS binding properties of these conjugates. We found that both of the LBP_{86-106}-IgG conjugates bound ^{3}H-LPS extremely well in aqueous buffers, but that binding was substantially decreased in the presence of serum. This decrease in binding was not due to instability of the conjugates in serum (as assessed by a lack of release of ^{14}C from the conjugates). Since a different peptide-IgG conjugate ($CAP18_{106-138}$-IgG) binds LPS well in serum and whole blood, and since $CAP18_{106-138}$ and another LPS binding peptide based on Tachypleus anti-LPS factor (TALF) contains a distinctive chemical structure that can be aligned to resemble a loop bent back on itself, we hypothesize that higher binding affinity in whole blood may be obtained by generating the same structure based on LBP (i.e. $LBP_{86-106-106-86}$). Our first attempt at obtaining this peptide failed, and we are in the process of repeating this synthesis.

While working with this series of LBP peptides, we evaluated the antibacterial activities of the peptides that we generated. These results, which were summarized in the last work report, indicated that LBP_{86-106} is an antibiotic. Furthermore, we found that the antibacterial activity of this peptide is markedly increased by the addition of a free cysteine on the carboxy terminal of the peptide. The antibacterial activity is maintained in whole blood that is anticoagulated with calcium chelating antibiotics. However, LBP_{86-106} with a terminal cysteine fails to kill E. coli in the same experiments performed in Teflon tubes in the absence of anticoagulants. We hypothesize that depletion of divalent cations in the bacterial cell wall results in destabilization of the adjacent LPS molecules, leading to enhanced antibiotic activity. Accordingly, we are hopeful that $LBP_{86-106-106-86}$ will have higher LPS binding activity, and that this activity will translate into increased antibiotic activity in the presence of whole blood.

Progress on specific aim #2

At the start of the grant, our forward progress with the LBP family of peptides led us to focus our energy on LBP and LBP peptide-IgG conjugates. Therefore, when we found that there was decreased binding activity of LBP_{86-106}-IgG in whole blood in the absence of anticoagulants, we began work on the elucidation of the LPS binding domain of BPI. This work has gone remarkably rapidly, both because the techniques have been developed and fine tuned for the study of the LBP peptides and because we were guided by prior work indicating that certain peptides in the amino terminus of BPI had biological activity (Little et al., 1994; J. Biol. Chem. 269:1865-1872). We followed essentially the same protocols as that outlined in the original grant application. This consisted of synthesizing overlapping peptides in the amino terminus that had likely LPS binding sequences, and then assaying the peptides by a capture slot blot assay for LPS binding affinity. These results are shown in a table at the end of this report, and indicate that the peptide $BPI_{85-99}C$ has the best binding affinity. Two interesting aspects of these data are notable. First, this sequence has marked similarity and homology to LBP_{86-106}. Second, this sequence with a cysteine on the carboxy terminal ($BPI_{85-99}C$) was extremely active. However, the same peptides without a cysteine (BPI_{85-99}), and with a cysteine on the amino terminal (C-BPI_{85-99}) are much less active. These findings are unexpected, unexplained, and novel.

We next coupled several of the generated BPI peptides, including $BPI_{85-99}C$, to human IgG to create BPI peptide-IgG conjugates. For these experiments we utilized the technology we developed earlier for the TALF, CAP18 and LBP peptide conjugates, and we used SMPT as the coupling agent in order to create a more stable conjugate than SPDP. These experiments again proceeded rapidly because of our prior experience from earlier experiments in the grant. We have started to evaluate the LPS binding activity of the generated constructs using the radioimmunoassay with ^{3}H-LPS, and these results are shown below. Not unexpectedly, the BPI peptide conjugates bound LPS in proportion to the LPS binding affinity of the peptides coupled to the IgG. Thus, BPI_{85-99C}-IgG bound LPS the best. Half-maximal binding of 5 μg/ml of E. coli O25 was achieved at approximately 30 μg/ml of BPI_{85-99C}-IgG. Half-maximal binding of a different LPS (E. coli O18) by this conjugate was obtained at 70 ug/ml.



Work on Specific aim #3

As noted in prior reports, we have developed techniques for using different heterobifunctional linkers (SPDP, SMPT, SMCC) to link LPS binding peptides to IgG. As above, we are now routinely using SMPT as a linker because we have shown that the complexes with SMPT remain stable in blood.

Work on Specific aim #4

This specific aim is to characterize the peptide-IgG conjugates as we develop (and modify) them. The methodology for most of these studies are now established. We now believe that the ability of a conjugate to promote opsinophagocytosis may be an important property for protection. Accordingly, over the last 3-4 months, we have set up an assay of opsinophagocytosis using peripheral blood mononuclear cells as a source of WBC and radiolabeled LPS as a means of following uptake of into the cells. Because LPS released from the bacteria (in its native physicochemical form) is of the most interest, we have gone to the extra difficulty of specifically radiolabeling bacteria in the LPS moiety by growing E. coli that contain the Gal E mutant in ^{3}H-galactose. Uptake of radiolabel is essentially exclusively into the LPS of the E. coli. For the initial assays, the radiolabeled bacteria were pre-incubated with normal IgG (as a negative control), IgG specific for the O-antigen of LPS (as a positive control), and a peptide-IgG conjugate consisting of a CAP18 peptide ($CAP18_{106-138}$-IgG). These mixtures were then incubated with WBC that had been purified over Ficoll. The cells were then washed, and counted for radioactivity in a beta scintillation counter. The results of two preliminary experiments are shown below.

Opsinophagocytosis assay: uptake of ^{3}H-LPS into peripheral blood WBC

Antibody	Exp. 1	Exp. 2
Normal IgG	615	603
Mouse anti-O	12,723	9,898
CAP18-human IgG	643	618
rabbit anti-human IgG	428	593
Normal IgG + rabbit anti-human IgG	628	698
CAP18-human IgG+ rabbit anti-human IgG	1713	1100

Since the entire bacterial/conjugate mixture was incubated with the WBC, these experiments studied the uptake of both ^{3}H-LPS released from the bacteria as well as ^{3}H-LPS in the bacterial wall. To evaluate if the IgG preparations altered the amount of LPS released, we performed parallel experiments that consisted of incubating the conjugates with the bacteria, after which the mixtures were centrifuged and the percent LPS released into the supernatant (the released LPS) and the percent remaining in the bacterial pellet (bacterial associated LPS) were calculated. The results of four experiments are shown at the top of the next page, and indicate that the CAP18-human IgG leads to a marked release of LPS from the bacteria.

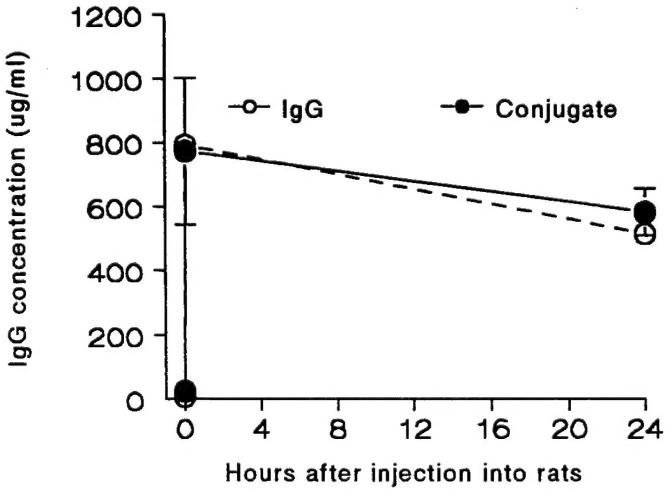
Release of ^{3}H-LPS

Antibody	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean ± SD
Normal IgG	13. 0	12.0	19.0	13.0	14.25 ± 2.68
Mouse anti-O	11.0	10.0	18.0	13.0	13.00 ± 2.87
CAP18-IgG	61.0	30.0	43.0	31.0	41.25 ± 5.86

Although the assays are still being developed, and the conjugates are still being evaluated, we believe that the assays will eventually allow us to measure the ability of the LBP and BPI conjugates to augment phagocytosis of bacterial associated and released LPS. In the initial experiments, however, the only conjugate studied ($CAP18_{106-138}$ peptide-IgG) failed to increase phagocytosis, despite the fact that it resulted in an increase in released LPS (presumably through its antibiotic effect). This same conjugate binds LPS with high affinity. Opsinophagocytosis of the conjugate was increased somewhat by adding anti-human IgG, which would supply an unaltered Fc portion to the LPS-conjugate complex, although not to the level of the anti-O IgG positive control. We believe that the results, if confirmed, may indicate that the Fc portion of the conjugate may be altered during the coupling. This seems a crucial issue for future study. A goal for the next trimester will be to test the LBP and BPI based conjugates in this system.

Work on Specific aim #5

We have started this specific aim, which is to assess the phamacokinetics of the conjugates in animals. In preliminary studies, we injected 2 rats with a $CAP18_{106-138}$-IgG and 2 rats with control IgG, each at a concentration of 10 mg/kg. We then studied the levels of IgG in serum obtained from the rats over a 24 hour period. These preliminary results are shown below, and indicate that there is no difference in the pharmacokinetics of peptide-IgG conjugates and control IgG.



Work on Specific aim #6-7

Because we do not yet have our optimal LBP and BPI constructs, we have not started animal experiments of *in vivo* LPS clearance or protection. However, we believe that we are close, and for this reason have started to make the arrangements for these studies. We will determine the doses for these experiments from the pharmacokinetic studies analogous to that shown above.

II. New Knowledge

1. Of the peptides studied, the sequence in the amino portion of BPI that has the highest affinity for LPS is $BPI_{85\text{-}99C}$. Addition of a cysteine to the carboxy, but not the amino, end of the peptide markedly increases LPS binding.

2. Peptide-IgG conjugates consisting of $BPI_{85\text{-}99C}$-IgG bind LPS with high affinity in buffer assessed by RIA.

3. The T½ of a peptide-IgG conjugates made with SMPT ($CAP18_{106\text{-}138}$-IgG) is not different from control IgG and is approximately 24 hours (for human IgG in rats). Presumably the T½ of peptide-IgG made with human IgG would be longer in humans.

4. A novel opsinophagocytosis assay has been developed that measures opsinophagocytosis of ^{3}H-LPS that is in the bacterial wall or released from dying bacteria. In preliminary studies, the SMPT conjugate $CAP18_{106\text{-}138}$-IgG kills bacteria, leading to increased release of ^{3}H-LPS from bacteria, but does not substantially increase opsinophagocytosis into peripheral WBC.

III. Technical problems

As noted above, we attempted to synthesize a modified second generation LBP peptide that is folded back on itself in order to further increase LPS binding activity. The initial attempt at obtaining this peptide ($LBP_{86\text{-}106\text{-}106\text{-}86}$) was not successful. Accordingly, we are repeating this effort on a different peptide synthesizer. We do not expect that this will be a difficult problem.

There have been no other technical problems.

IV. Budget

As noted in the last project summary, we are running approximately 4 months behind our initial anticipated spending budget. As noted, this is due to a delay in starting the project at the

outset at the start of 1994 that has been carried forth to the present. I would like to reaffirm and emphasize that this delay in spending reflects a delay in starting the project and billing that has not yet caught up with actual spending rather than the fact that we are not intensively working on the project (as hopefully these project summaries indicate).

V. Publications

We have written three articles that have been or are on the verge of submission:

1. Fletcher MF, Kloczewiak, Loiselle PM, Amato SF, Black KM, Warren HS. TALF peptide-immunoglobulin G conjugates that bind lipopolysaccharide. Submitted.

2. Fletcher MF, Kloczewiak M, Loiselle PM, Ogata M, Vermeulen MW, Zanzot EM, Warren HS. A peptide-IgG conjugate, $CAP18_{106-138}$-IgG, that binds and neutralizes endotoxin and kills Gram-negative bacteria. In final preparation for submission.

3. Ogata M., Kloczewiak M, Fletcher MF, Loiselle PM, Zanzot EM, Vermeulen MW, Warren HS. The effect of anticoagulants on LPS binding and neutralization by the peptide immunoglobulin conjugate, $CAP18_{106-138}$-IgG in whole blood. In final preparation for submission.

VI. Future directions

We are following our initial outlined proposal essentially as written. Specific aim #2 is now largely complete. In addition, we now have first generation BPI peptide-IgG conjugates in hand, and we are beginning studies of their characteristics and functional abilities. Our general goals for the next trimester will be to assess the ability of the LBP conjugates that we have in hand to neutralize TNF in whole blood, to synthesize and generate the second generation LBP peptide and conjugate ($LBP_{86-106-106-86}$ and $LBP_{86-106-106-86}$-IgG), and to evaluate if these "double backed peptides increase activity. We will follow our outlined plan to work up and test the BPI conjugates. We plan to continue to modify the opsinophagocytosis assay, and to evaluate the LBP and BPI conjugates in this assay. Because of the expense of these studies, we plan to await a conjugate that fulfills all of our ideal criteria before scaling up and studying it in animals. This will hopefully occur in 3-6 months.

Specific goals for the next trimester include:

1. synthesis of $LBP_{86-106-106-86}$ and generation of $LBP_{86-106-106-86}$-IgG
2. binding studies of $LBP_{86-106-106-86}$-IgG
3. characterization and binding studies of BPI peptide conjugates
4. antibiotic profiles of BPI peptides and conjugates
5. TNF inhibition studies of LBP and BPI conjugates that are currently available
6. continuation of opsinophagocytosis studies; evaluation of LBP and BPI conjugates
7. polish and submit LBP peptide paper

Table 1 - Binding of Lipopolysaccharide (LPS) to Peptide Analogues of BPI: Minimal Peptide that Binds LPS.

	Mean +/- S.D. (nanograms)		Mean +/- S. (picomoles)	
NH4-M V P N V G L K F S I S N A N I K I S G K W K A Q K R F L K M S G N F D L S I E G -COOH				
M V P N V G L K F S I S N A N	>2000	-	>1257	-
G L K F S I S N A N I K I S G	145	53	93	34
G L K F S I S N A N I K I S G C	258	97	156	59
I S N A N I K I S G K W K A Q	>2000	-	>1206	-
I K I S G K W K A Q K R F L K	>1733	-	>946	-
C I K I S G K W K A Q K R F L K	467	41	241	21
I K I S G K W K A Q K R F L K C	130	83	67	43
K W K A Q K R F L K M S G N F	>1733	-	>927	-
K R F L K M S G N F D L S I E	850	595	476	333
M S G N F D L S I E G M S I S A	Peptide insoluble			

n = 3